Cellulolytic and lactic acid bacteria in the gastro–intestinal tract of the horse

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Summary

The conditions in various segments of the GI tract of the horse determine the population sizes and types of microbes that reside there. Acidity and a short digesta residence time in the stomach select for acid–tolerant and fast–growing bacteria, of which lactic acid producing bacteria are the most numerous. In the hindgut, the neutral pH (pH≥7.0), availability of fibre and the long residence time of digesta support a diverse bacterial population. Fibre–digesting bacteria predominate in the hindgut when the diet contains high levels of fibre. However, a change from a normal fibre–rich diet to a starch–rich diet will alter the bacterial population and the end products of bacterial fermentation in the hindgut. When starch supply is plentiful, the amount of volatile fatty acids and lactic acid produced may exceed the capacities of the GI tract and associated body tissues to absorb and metabolize them. This can lead to development of fermentative acidosis and laminitis. Further knowledge about the microbial population in the GI tract of the horse would assist nutritionists and veterinary practitioners to develop better dietary practices.

Keywords: 16S rDNA, DGGE, cellulolytic bacteria, acidosis

Introduction

The size and diversity of the microbial population in the gastrointestinal (GI) tract of the horse is the product of a very long and complex evolutionary process that has occurred over millions of years. These microbes aid the digestion of feed, and the host provides the physiological and nutritional factors (e.g., substrate, pH, osmotic pressure) critical for the maintenance and growth of the microbial population (Goodson *et al*. 1988). Dietary changes that affect theseparameters can lead to profound changes in the microbial population. The microbes interact with the host animal directly and indirectly, and with each other, making this ecosystem extremely complex. The evolutionary process has produced the horse as an animal unique to its environment with a modified digestive system well suited for the utilisation of the fibrous feed it consumes. As a simple stomached herbivore having an enlarged caecum and colon, the horse is able to extract most of the digestible nutrients from the diet before the digesta reaches the hindgut. In the hindgut, digesta is retained for 6–12 h in the caecum, 24–50 h in the large colon and 8–12 h in the small colon (Kohnke *et al*. 1999). These retention times allow the microbes to extract energy from residual structural carbohydrates, thereby contributing to the total energy economy of the horse. Under normal feeding conditions, the fermentation of structural carbohydrates is a slow process and the absorption of volatile fatty acids (VFAs) from the caecum and colon is very efficient (Argenzio *et al*. 1974). However, the balance between VFA production and absorption may change if a fibre–deficient diet rich in starch or soluble carbohydrates is fed. The small intestine of the horse has limited capacity to digest starch: the amount of amylase produced is only 8–10% of the amount produced by the pig (Jackson 1998). It has been suggested that the maximum level of starch that can be digested in the small intestine of the horse is approximately 3.5 $g \cdot kg^{-1} \cdot BW$ (Potter *et al*. 1992). However, factors such as the amount and source of starch ingested and the source of fibre in the diet explain some of the variation in reports on the capacity of the small intestine to digest starch (Kienzle 1994). Excess starch that is not digested in the small intestine enters the caecum, where it is rapidly fermented, resulting in the production of large amounts of organic acids, particularly lactic acid. Conditions in the caecum and the colon of the horse are similar to those in the reticulo–rumen (Sneddon and Argenzio 1998). The fermentation of starch can also be extensive in the non–glandular section of the stomach, where lactic acid producing bacteria (LAB) are present in large numbers (about 10^9 CFU/g stomach content; de Fombelle *et al*. 2003). Lactic acid produced in the stomach and the hindgut is absorbed into the

bloodstream and may cause systemic acidosis. Lactic acid may also be a pre–disposing factor for the inflammatory process that produces laminitis (Garner *et al*. 1977).

Little is known about the bacterial community in the GI tract of the horse, particularly the cellulolytic bacteria. Previous studies using culture–dependent techniques have failed to detect the presence of a diverse population of cellulolytic bacteria, and only a limited number of species has been identified. Three species were identified: *Ruminococcus flavefaciens* was identified as the predominant bacterium and *Fibrobacter succinogenes* and *Ruminococcus albus* were found at low numbers in all of the animals studied by Julliand *et al.* (1999). Sequence analysis of 272 random clones generated from bacterial DNA extracted from samples of the large–intestinal wall tissue and lumen contents from grass–fed horses revealed a diverse bacterial population, as the sequences showed low similarity with existing entries in the EMBL–GenBank databases (Dally *et al.* 2001). Only 13 (5%) of these 272 clones seemed to represent previously described bacteria, e.g., *Streptococcus bovis, R. flavefaciens, Clostridium barati, Butyrivibrio fibrosolvens, Lactobacillus salivarius,* and *Campylobacter lanienae*. In another study based on 16S rRNA–targeted oligonucleotide probes, it was estimated that *F. succinogenes* represents about 12% of the bacterial population in the caecum, and about 4% of that in the colon (Lin and Stahl 1995). Kioke *et al.* (2000) reported a seasonal variation in cellulolytic bacterial species in the caecum of Hokkaido native horses: *F. succinogenes* was predominant in winter and *R. albus* and *R. flavefaciens* were predominant in summer. Hokkaido native horses are able to survive very cold and harsh winter conditions by consuming bamboo grass and bark. Under these conditions, other horses can hardly maintain body weight. The high population of *F. succinogenes* was therefore believed to contribute most to the digestion of structural carbohydrates in the caecum and the colon of the horse (Kioke *et al*. 2000).

In comparison with cellulolytic bacteria, work on lactic acid producing bacteria has been more extensive, particularly in relation to the metabolic disorders, fermentative acidosis and laminitis. *Streptococcus bovis* and *S. equinus* are the predominant lactic acid producing bacteria in the caecum and colon of the horse (Al Jassim and Rowe 1999). Other LAB found in the GI tract of the horse were closely related to *Lactobacillus salivarius*, *Lactobacillus mucosae*, *Lactobacillus delbrueckii* and *Mitsuokella jalaludinii*. The contribution of some of these bacteria to lactic acid build up in the hindgut of the horse has been established (Al Jassim *et al*. 2005). Certain species of LAB are of special interest, as they are believed to be beneficial to the animal and are promoted as probiotics. It is important to note that under normal feeding conditions, LAB are only present in low numbers and cause no harm to the horse. However, when starch becomes the main constituent of the diet, significant amounts of starch are fermented in the hindgut, which may result in lactic acid accumulation, acidosis and laminitis.

Under pasture conditions, horses graze for approximately 16–18 hours each day (Kohnke *et al*. 1999). Fibre is the main dietary constituent of the feed under grazing conditions. Pasture also supplies the horse with its dietary requirements for protein, minerals and vitamins. Horses ingest their feed at a much slower rate than ruminant herbivores, but chew the feed thoroughly prior to swallowing. Thus, feed enters the stomach as small particles saturated with saliva, to undergo the preparatory phase of digestion. The saliva contains no enzymes but has buffering capacity provided by bicarbonate (about 3 g L^{-1}), which maintains mild acidic conditions at the entrance to the stomach (pH = 5.4). This favours the growth of lactic acid producing bacteria, which proliferate when the diet is composed of readily available carbohydrates. Starch from cereal grains, and soluble sugars from temperate grasses support the growth of LAB and increase the production of lactic acid.

This paper presents recent findings on the diversity of cellulolytic and lactic acid producing bacteria in the GI tract of the horse. The data are derived from molecular and culture–dependent techniques. The population of cellulolytic bacteria was characterized by denaturing gradient gel electrophoresis (DGGE) performed using enriched caecal bacterial cultures grown with various cellulose sources. Lactic acid producing bacteria were isolated from various segments of the GI tract and analysed for production of VFA and L– and D–lactic acid. LAB were cultivated using a modified semi–selective MRS–agar medium and bacteria were identified by comparing 16S rDNA sequences with entries in the EMBL–GenBank databases.

Experimental procedures

Two attempts were made to isolate cellulolytic bacteria from the GI tract of the horse. In the first attempt, contents from the caecum, colon and rectum were collected from two horses post mortem. The samples were processed under CO_2 and the cellulolytic bacteria were cultured in tubes with basal medium 10 (BM10) supplemented with strips of filter paper (Caldwell and Bryant 1966). The tubes were incubated for 21 days and were scored positive if there was any visual breakdown of the cellulose strips. Most probable number calculations were then used to estimate the numbers of cellulolytic bacteria (Garthright 2001). In order to isolate the cellulolytic bacteria, tubes that scored positive were vortexed for 30 s and 1 mL of bacterial culture was serially diluted ten–fold up to 10^{-7}. Three dilutions (10^{-5}, 10^{-6} and 10^{-7}) were used to inoculate roll tubes containing BM10 agar medium supplemented with glucose and cellobiose (0.1% of each). After incubation for 48 h at 39°C, ninety–six colonies were picked and inoculated into a broth of BM10 with glucose (0.2%). After another

48 h of incubation, the culture was examined under the microscope to check the purity, morphology and Gram staining characteristics. The pure isolates were then tested for their ability to degrade filter paper and ferment other carbohydrate sources. Eighty pure isolates were differentiated by restriction fragment length polymorphism (RFLP) analysis (Krause *et al*. 1999) and 39 representative isolates were identified by 16S rDNA sequencing.

In the second attempt, pooled caecal contents were collected from three groups of six horses. Samples were kept warm during transport to the laboratory and were continuously flushed with CO_2. Samples were then flushed with CO_2 and 10 g samples were placed in sterile stomacher bags containing 90 mL of anaerobic dilution solution (ADS). The contents were flushed with CO_2 again and homogenized in a stomacher (Stomacher 400 Circulator, Seward Ltd., Thetford, UK) for two cycles of 30 s at 230 rpm. The samples were then strained through a four–layer sterilized cheesecloth and serially diluted ten–fold with ADS to a final dilution of 10^{-8}. Four dilutions (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}) were inoculated in triplicate into tubes containing BM10 broth supplemented with a fibre–carbon source of cotton thread, filter paper, or neutral detergent fibre (NDF) as described by Caldwell and Bryant (1966). The tubes were incubated at 39°C and observed regularly for fibre breakdown during the next 21 d.

After complete breakdown of cellulolytic material, cultures were centrifuged and the DNA pellet was extracted and stored at –80°C until analysis. Because ten selective media tubes (BM10 supplemented with cotton thread, filter paper or NDF) exhibited complete or partial breakdown of media over a 21–day period, this period was used for further assays.

Total DNA was extracted from 9 mL cultures using centrifugation, lysis buffer and bead beating. PCR was performed to amplify the 16S rDNA in preparation for DGGE analysis using primers 341F + GC and 518R (Muyzer *et al*. 1993). Samples were then run on a DGGE for 18 h followed by silver staining. Sixteen bands were chosen for further study. These bands were cut out for further DNA analysis and sequencing. The DNA of the gel plugs was amplified as described previously for pre–DGGE PCR, and then subject to electrophoresis using a 2% agarose gel. Purified PCR products were then ligated into pGEM–T Easy vector (Promega, Madison, USA) and transformed into *E. coli* Top10 cells. Sequencing was performed on five transformed colonies using the T7 primer found within the pGEM–T vector.

The procedures for isolation and identification of lactic acid producing bacteria were described earlier (Al Jassim *et al*. 2003). The isolates were obtained from various segments of the GIT of horses exposed to dietary regimens such as roughage, roughage plus grain and oligofructose (Raftilose) dosing (Al Jassim *et al*. 2005). Genomic DNA was obtained from each isolate and the 16S rDNA amplified by PCR. The diversity of LAB isolates was initially determined by RFLP analysis, and selected isolates from each RFLP group were cloned and sequenced. A more definitive analysis of the population diversity was undertaken by comparing the 16S rDNA sequences of the isolates from this study with those found in public databases. *In vitro* production of L– and D–lactate and VFAs was also determined for each isolate.

Results

Fermentation in various parts of the digestive tract

Supplementary feeding of dry–rolled (DR) and steam–flaked (SF) sorghum to medium quality grass hay diets (*Urochloa panicoides*) resulted in high levels of L– and D–lactic acid in the stomach, particularly in the non glandular section (Figure 1); the SF sorghum resulted in a lower pH in the stomach (results not shown). Starch intake for the DR and SF sorghum diets was approximately 3.26 $g \cdot kg \cdot BW^{-1} \cdot meal^{-1}$, which is slightly below the maximum for small intestine digestion (Potter *et al*. 1992). Lactic acid produced in the stomach was mainly absorbed before digesta reached the caecum. In contrast to the stomach, fermentation in the hindgut produced SCVFAs, primarily acetic, propionic and butyric acids. The concentration of lactic acid in the hindgut was low, indicating that little starch entered the caecum. The pH of digesta in the remainder of the GI tract ranged between pH 6 and pH 7, except for that of digesta in the duodenum, which was slightly below pH 6.

Cellulolytic bacteria

Isolates picked from roll tubes were typically Gram–negative or Gram–positive, and no single type predominated. Bacterial numbers in the caecum, colon, and rectum averaged 1.27×10^8, 3.05×10^8 and 1.85×10^8, respectively, and the morphology of bacteria differed between sites. Isolates from the caecum were mainly Gram–negative curved rods, those from the colon were mainly Gram–positive cocci and those from the rectum were mainly Gram–negative short rods. None of the isolates consisted of cellulolytic bacteria.

In the second attempt, 16 bands selected from the DGGE gels were cloned and sequenced (Figure 2). Most bands were present in samples from all treatments. Band 1 from the NDF enriched sample (Figure 2) was 100% similar to that of the cellulolytic bacterium, *Ruminococcus flavefaciens*. Two bands of particular interest are bands 13 and 14 from enrichments with cotton thread (Figure 2, lane A2). These bands were not observed when samples from the same animal group were enriched with filter paper or NDF. These bands disappeared when the enriched cotton thread culture was subcultured onto NDF. The sequence data for these bands indicate that they are genetically related to unidentified rumen isolates and a *Clostridium* isolate. The 16S rDNA sequences of the clones were placed into a phylogenetic tree with known isolates (Figure 3).

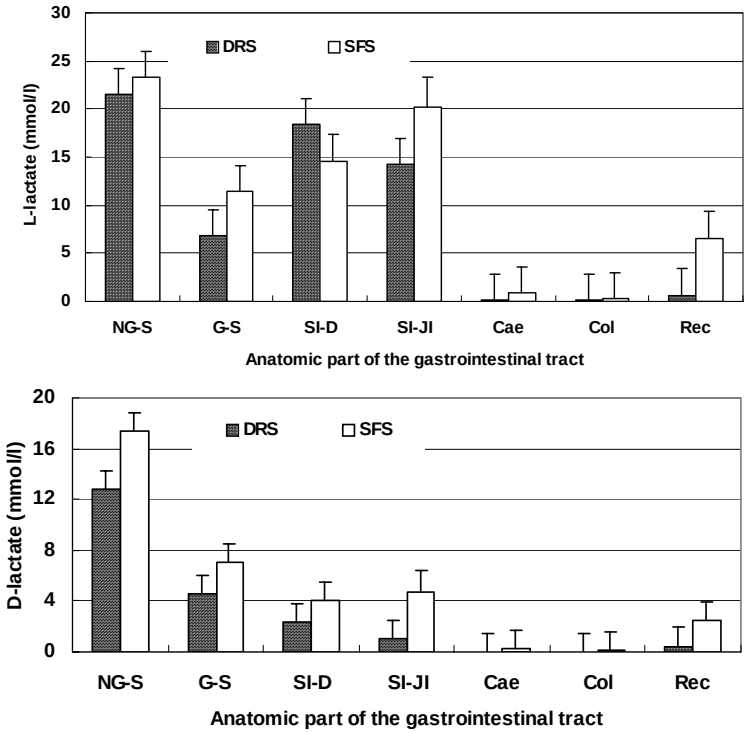

Figure 1 Effect of supplementation of liverseed grass (*Urochloa panicoides*) hay with dry rolled or steam flaked sorghum grains on the concentrations of L– and D–lactate in various parts of the gastrointestinal tract (Al Jassim 2005). DRS, dry–rolled sorghum; SFS, steam–flaked sorghum; NG–S, non–glandular region of the stomach; G–S, glandular region of the stomach; SI–D small intestine duodenum; SI–JI, small intestine jejunum and ileum; Cae, caecum; Col, colon; Rec, rectum.

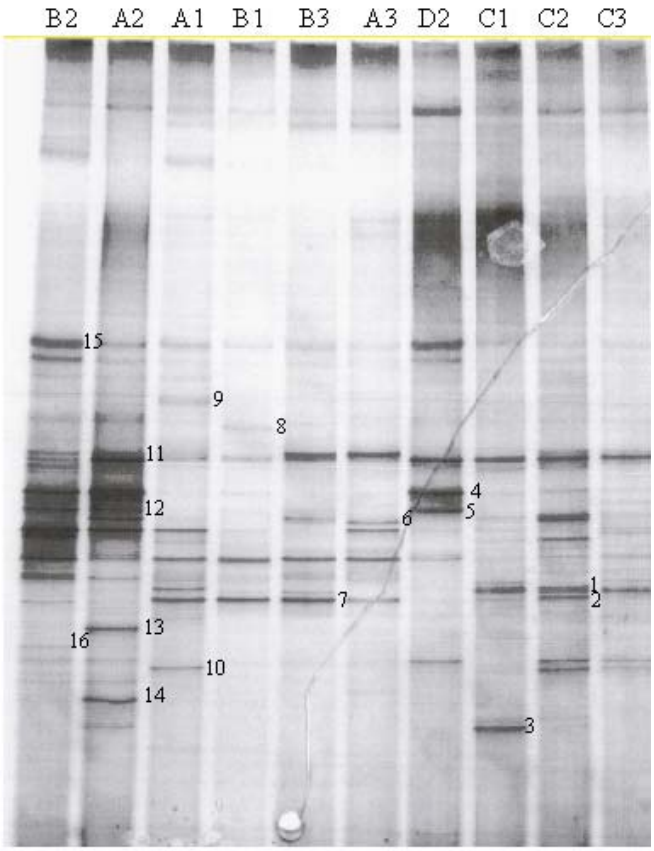

Figure 2 DGGE analysis of the PCR–amplified variable III region of the 16S rRNA gene from samples enriched on either (A) cotton thread, (B) filter paper, (C) NDF, (D) cotton thread and then NDF. Numerals appended enrichment treatment abbreviations represent samples from pooled animal groups. Numbers to the right of bands correspond to bands picked for sequencing.

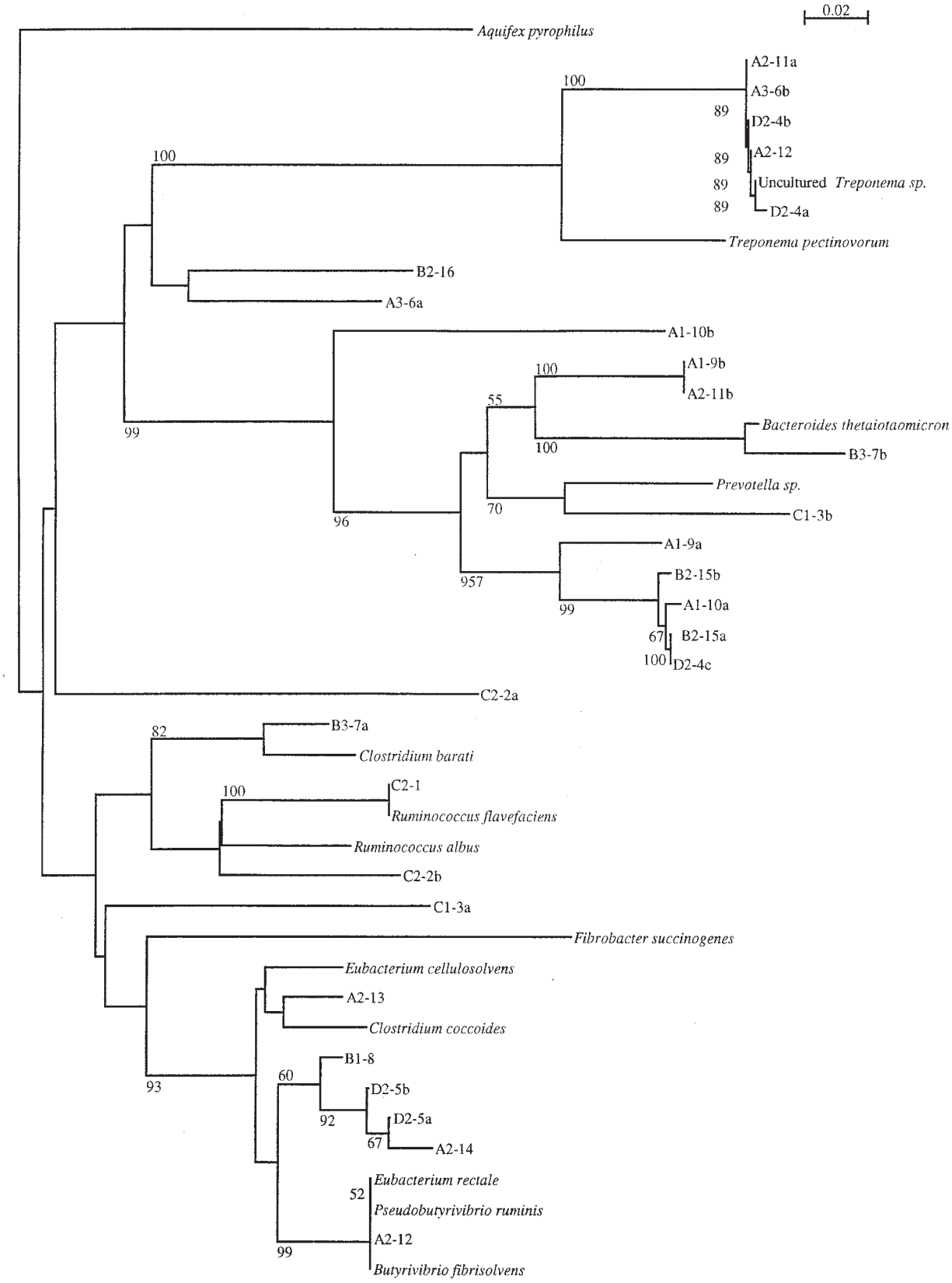

Figure 3 Phylogenetic relationships of derived sequences from the 16S rRNA variable III region constructed using the neighbour–joining method. The bootstrap resampling values for 16S rRNA gene sequences (expressed as percentages) from 1000 resamplings are indicated. Bootstrap values of less than 50% are not depicted. Codes for clones are composed of three parts: (1) A = cotton thread, B = Filter paper, C = Neutral detergent fibre (NDF), D = initial enrichment on cotton thread and then sub–cultured onto NDF; (2) 1 = animal group 1, 2 = animal group 2, 3 = animal group 3; (3) this numeral denotes the band number from the DGGE gel analysis and a letter suffix indicates instances in which different clones were identified from the same band.

The most numerous group of sequences identified appear to be uncultured bacterium clones from the gastrointestinal tracts of humans (7), pigs (4), chickens (1), termites (4), and from waterways (1), the deep sea (1) and the rumen (1). Other sequences showed >97% correlation to database sequences for the genus *Clostridium* (3), *Ruminococcus flavefaciens* (1), *Fusobacterium necrophorum* (1), *Clostridium hathewayi* (3), *Pseudobutyrivibrio ruminis* (4) and *Prevotella* spp. (2). The sequence of band 11 from the cotton thread enrichment (this band was observed in all samples) was found to have highest similarity to an uncultured *Treponema* sp., although the similarity was only about 90%.

Lactic acid producing bacteria

Isolates from the rectums of horses fed pasture, grass or lucerne hay supplemented with grain were primarily Gram–positive bacteria that were very closely related (>99%) to *S. bovis* and *S. equinus*. These two species are 100% identical in their 16S rDNA sequences, but differ in their ability to hydrolyse starch. Although *S. bovis* has a strong affinity for starch, *S. equinus* lacks the ability to hydrolyse starch but grows well on the end products of starch hydrolysis (maltose and glucose), producing L–lactate but not D–lactate. Other isolates were obtained from horses fed hay or hay plus grain and from horses given an oligofructose challenge. Lactic acid producing bacteria other than *S. bovis* and *S. equinus* were studied.

16S rDNA was amplified from 25 isolates of LAB and subjected to RFLP analysis prior to sequencing. Using the restriction enzymes *Hinf*I, *Hha*I and *Mbo*I, 18 distinct 16S rDNA RFLP patterns were identified and representatives from each RFLP group were subjected to near complete 16S rDNA sequence analysis. Thirteen of the isolates were closely related to species from the genus *Lactobacillus* and clustered into three groups (Figure 4). Group 1 included four isolates: one from the stomach (RA2053) and three from the caecum (RA2070, RA2071 and RA2087). This group shared from 95% to more than 99% identity with *L. mucosae*. Group 2 included four isolates: two from the stomach (RA2062 and RA2066) and two from the rectum (RA2113 and RA2120), and clustered with *L. delbrueckii* at 96–98% identity. Group 3 had five isolates, three of which were closely related to *L. salivarius* (>99% identity). One of these isolates (RA2105) originated from the colon, and two were from the rectum (RA2115 and RA2116). The two remaining isolates in group 3 (T057 and T059) were from the stomach and shared only 94% identity with *L. salivarius*. Four of the remaining five isolates were related to *Mitsuokella jalaludinii* with 97–98% identity. Two of these isolates (RA2074 and RA2083) were from the caecum and two were from the rectum (RA2107 and RA2108). The remaining isolate was from the rectum (RA2114), and clustered with *Veillonella atypica* but with only 93% identity (Figure 3).

Discussion

The degradation of filter paper strips during the first enrichment and the subsequent failure to obtain pure cellulolytic bacterial isolates from roll tubes during the secondary enrichment step support the results of earlier studies, which show that cellulolytic bacteria grow slowly and that those of equine origin require a longer lag phase to initiate digestion of substrate (Sunvold *et al*. 1995, Koller *et al*. 1978). It is likely that cellulolytic bacteria were out–competed by other fast growing strains during the long lag phase period and in the presence of soluble carbohydrates such as those in the roll tube media. The difficulties of obtaining pure cultures of cellulolytic bacteria from the digestive tract are widely acknowledged. Hungate (1966), who isolated cellulolytic bacteria using BM10 agar roll tubes supplemented with ball–milled cellulose as the only carbon source, often found that the cellulolytic isolates were contaminated with non–cellulolytic bacteria. Pure cultures were obtained by diluting out the contaminants. A different approach was adopted by Bryant and Burkey (1953) in which they isolated pure cultures on soluble carbohydrates then tested the isolates for their ability to degrade cellulose. We followed this approach in our study but failed to obtain pure cultures of cellulolytic bacteria. 16S rDNA directed PCR primers targeted at *F. succinogenes* confirmed the presence of this bacterium in the rumen of cattle but not in the caecal contents of the horse. Previous work by Lin and Stahl (1995) demonstrated that *F. succinogenes* of equine origin is phylogenetically different from that of bovine origin. A preference for cotton thread cellulose has also been reported for *F. succinogenes* (Hespell *et al*. 1997). Other studies have demonstrated that the majority of bacterial clones from the equine gut do not correspond to any of the entries recorded in the EMBL–GenBank databases (Daly *et al*. 2001). It was found that 77% of equine bacteria sequences showed similarity to previously characterised bacteria (90–97% homology), while 32 sequences (12%) showed less than 90% homology to their nearest database entry (Daly *et al.* 2001). This result compares favourably with the DNA sequence analysis of the DNA sequences of the bacteria isolated during the second enrichment.

LAB are easier to culture than cellulolytic bacteria. However, the use of MRS medium to enumerate LAB may over–estimate numbers of culturable, viable cells. Although MRS medium was designed to enumerate bacteria belonging to the *Lactobacillus*, *Streptococcus*, *Leuconostoc*, and *Pediococcus* genera, use of this medium in our laboratory has shown that it also supports the growth of bacteria belonging to other genera such as *Escherichia*, *Clostridium*, *Selenomonas*, *Mitsuokella* and others. The majority of LAB identified by 16S rDNA sequencing belonged to several species of the *Lactobacillus* genus. The isolation of lactobacilli from different sections of the gastrointestinal tract of laminitic horses suggests that these bacteria have the

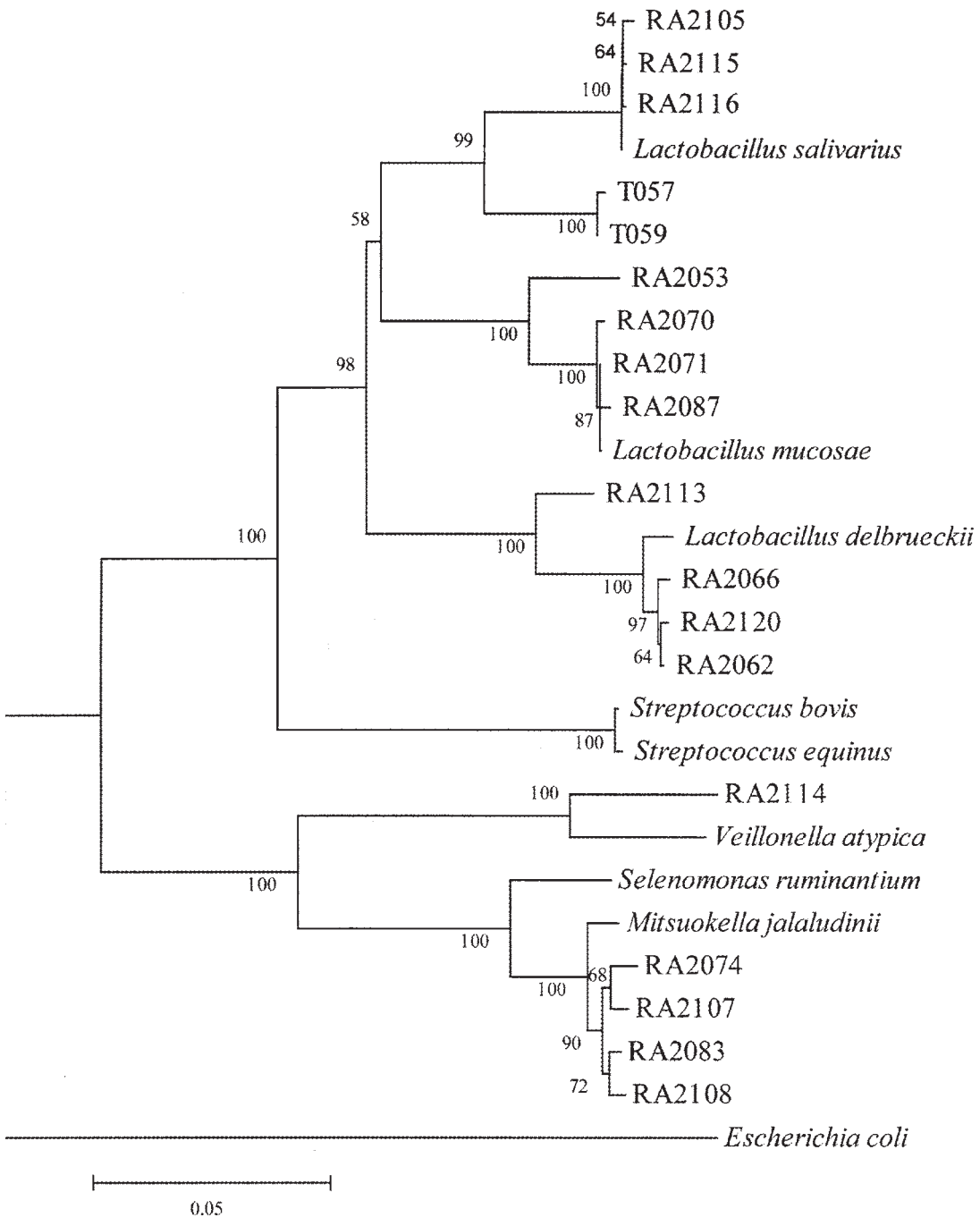

Figure 4 Phylogenetic relationship of LAB isolates from the equine gastrointestinal tract based on near–complete 16S rDNA sequence data. Bootstrap values were based on 1000 replications and are expressed as percentages.

ability to adapt to a range of tissue types and changes in pH. *Lactobacillus salivarius* and *L. mucosae* were the predominant lactobacilli identified. Our cultures of *L. salivarius* from the colon and rectal contents as well as the stomach demonstrate the potential of this bacterium to exist in all parts of the GI tract and indicate that it may contribute to lactic acid accumulation in the stomach and hindgut. Of particular interest are the D–lactate producers that shared between 97–98% sequence identity with *M. jalaludinii*, a bacterium first isolated from the GIT of cattle in Malaysia. These isolates have not been identified previously in the gastrointestinal tracts of horses. This level of sequence identity is on the borderline for assigning these isolates to the species *M. jalaludinii*. Further genetic and phenotypic characterisation, including percentage DNA–DNA relative reassociation with *M. jalaludinii* and other closely related species, will clarify the taxonomic designation of these isolates.

Unlike *S. bovis* and *S. equinus*, *Mitsuokella* spp. are Gram–negative bacteria and are therefore not sensitive to antibiotic feed additives such as

virginiamycin, which is used to prevent acidosis and laminitis in horses (Rowe *et al*. 1994; Al Jassim and Rowe 1999). This suggests that there will be situations where the use of virginiamycin may not provide full protection against acidosis in the horse. It will then become important to find alternative methods of control to identify conditions that favour the proliferation and establishment of *M. jalaludinii* in the gastrointestinal tract of the horse.

The findings reported in this paper may contribute to a better understanding of the microbial ecosystem of the equine gastrointestinal tract and may enhance our ability to monitor microbial changes in the GI tract and consequently the health and the wellbeing of the horse. DGGE analysis of the 16S rRNA PCR amplified variable III region is a useful tool for the visualization of bacterial population composition within mixed or environmental samples. Analysis was able to identify the presence of one of the most common cellulolytic bacteria found in the ruminant. Not all bands were sequenced but PCR amplification of the samples with primers specific for *F. succinogenes* also revealed the presence of this cellulolytic bacterium in all enrichment cultures (data not shown). Of particular interest are bands 13 and 14, which were only present in cotton thread enrichments and were not maintained when cultures were transferred to NDF media. Further investigation of these clones may indicate an inability to grow in the presence of NDF or filter paper but an ability to flourish in the presence of cotton thread. Another cluster of interest is that which exhibits 90% similarity to the spirochete, *Treponema.* The clones from band 11, which were observed in all samples and were predominant members within these populations regardless of enrichment media, are of particular interest. Further investigations may prove fruitful in identifying a role for these organisms with respect to fibre digestion.

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